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Zoological Research

Growth Differentiation Factor-9 Gene Expression of Mice Oocytes *in Vitro* and *in Vivo*

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Abstract: Mice preantral follicles were cultured *in vitro* for 12 days to achieve metaphase Ⅱ (MⅡ) oocytes. Oocyte growth differentiation factor-9 (GDF-9) gene expression was measured during different growth stages to explore the relationship between oocyte maturation and GDF-9 gene expression. Preantral follicles of 10-day old mice were isolated from the ovaries and were cultured for 12 days. Oocytes from day 2 (D2), D4, D6, D8, D10, D12 cultured *in vitro* were named the *in vitro* group and oocytes of day 12 (D12), D14, D16, D18, D20, D22 grown *in vivo* were named the *in vivo* group. Follicle survival, antrum formation and maturation rate were 89.5%, 51.8% and 56.6% respectively in follicles cultured *in vitro*. After RT-PCR and agarose gel electrophoresis, relative mRNA abundance of GDF-9 was measured in each group of oocytes. At day 8 – 12, the GDF-9 gene expression level of oocytes *in vitro* was significantly lower than that *in vivo* ($P < 0.05$). We conclude that MⅡ oocytes can be obtained from *in vitro* culture of preantral follicles. The GDF-9 gene expression of oocytes varies at different growth stages *in vivo*. The low expression of GDF-9 in oocytes cultured *in vitro* may be the cause of their low developmental capacity.

Key words: Mice; Culture; Oocyte; Growth differention factor-9

体外培养小鼠卵母细胞及其生长分化因子 – 9 基因表达

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摘要: 体外培养小鼠的窦前卵泡以得到第二次减数分裂中期(MⅡ)卵母细胞, 比较体外发育卵母细胞与体内生长的卵母细胞生长分化因子 – 9(GDF-9)的基因表达量, 探讨 GDF-9 的表达对卵母细胞体外发育成熟的影响。选择体外培养第 2 天(D2)、D4、D6、D8、D10、D12 卵母细胞作为体外发育组; 同窝雌性小鼠出生后 D12、D14、D16、D18、D20、D22 卵母细胞作为体内发育组; 半定量逆转录多聚酶链反应技术分别检测两组 MⅠ卵母细胞 GDF-9 基因表达量。结果体外培养小鼠窦前卵泡可以得到 MⅡ期卵母细胞, 卵泡成活率、窦腔形成率、卵母细胞成熟率分别达到 89.5%、51.8%和 56.6%。小鼠卵母细胞 GDF-9 基因表达量随发育时间的改变而发生变化, 而体外发育 D8—12 卵母细胞 GDF-9 表达量显著低于同期体内发育卵母细胞($P < 0.05$)。体外发育 D8—12 卵母细胞 GDF-9 基因表达量低于同期体内发育的卵母细胞的原因之一可能是其发育潜能较低。

关键词: 小鼠; 培养; 卵母细胞; 生长分化因子 9

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The interaction of oocytes and their associated follicle cells are very important for oocyte growth and development (Matzuk et al, 2002). Oocytes might regulate functions of granulosa cells in a paracrine way and consequently influence follicle growth and oocyte maturation (Gilchrist et al, 2004). Growth differentiation factor-9 (GDF-9), a member of the transforming growth factor β superfamily, is released from oocytes from the primary follicle period. GDF-9 contents varied greatly at different growth stages of oocytes (Elvin et al, 1999a). GDF-9 was essential for follicle growth because it could mimic some functions of oocytes and maintain the follicle structure. GDF-9 knocked-out mice were infertile and follicles could not grow over the

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3a stage (Elvin et al, 1999b), because the recruitment and proliferation of granulose cells and theca cells were blocked (Dong et al, 1996). GDF-9 in culture media helped vacuolated follicles grow in a spherical shape and induced the cumulus expansion when the oocytes were removed. In vacuolated follicles, luteinization inevitably occured (El-Fouly et al, 1970; Eppig et al, 1998). GDF-9 could inhibit the premature luteinization of follicles especially at later growth stages (Yamamoto et al, 2002) as it can inhibit LH receptor synthesis and progesterone and estrediol production in granulosa cells (Vitt et al, 2000). The roles of GDF-9 during oocyte growth and maturation are not completely known yet. We aimed to measure the relative abundance of GDF-9 mRNA in oocytes grown *in vitro* and *in vivo* at different points to explore the change of GDF-9 gene expression and its potential roles during oocyte growth and maturation.

1 Materials and Methods

1.1 Animals and reagents

Ten-day old Kunming female mice were housed with their mothers in an environmentally controlled room. Animals were bred according to the national standards for animal care.

The reagents used were: Minimal essential medium alpha (α-MEM, GIBCO), recombinant human follicle stimulating hormone (rFSH, Sereno), insulin, transferrin, selenium, foetal bovine serum (FBS), penicillin, streptomycin (Sigma), lysis buffer (0.5% NP-40, 10 mmol/L tris-HCl pH 8.0, 10 mmol/L NaCl, 3 mmol/L $MgCl_2$), superscript Ⅲ reverse transcriptase enzyme, trizol, poly (T) primers, 100 bp DNA marker, RNase inhibitor (Invitrogen).

1.2 Follicle isolation and culture

Mice were killed by the cervical dislocation method and their ovaries were delivered to the laboratory immediately. Early preantral follicles were mechanically isolated with fine needles under an stereoscope from the ovaries of 10-day old mice. Follicles with a diameter between 80 and 110 μm were selected and placed in the α-MEM drops in a culture dish overlaid with mineral oil. The medium and oil were balanced overnight beforehand. Two to four follicles were placed in the 20 μL of media drops. Culture media was supplemented with 0.1 U/mL recombinant human FSH, 5 μg/mL insulin, 2.75 μg/mL transferrin, 2.8 ng/mL selenium, 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin. The follicles were incubated at 37 ℃ in 5% CO_2 for 12 days. Media was changed by refreshing half (10 μL) of the media every two days. At day 12 of culturing, follicles were treated with 2.5 IU/mL HCG. Mucified cumulus oocyte complexes were collected 16－20 h later and oocytes were denuded from companion cumulus cells by repeated pipetting. Oocyte nuclear maturation was evaluated under an invert microscope.

1.3 GDF-9 mRNA analysis by semi-quantitative RT-PCR in oocytes grown *in vivo* and *in vitro*

Ten oocytes per day were collected from the *in vitro* cultured follicles at day 2, 4, 6, 8, 10, 12 (D2－D12; the *in vitro* group), cumulus cells were removed completely from oocytes by pipetting with 0.1% hyaluronidase and washed three times with PBS. In the same way, ten oocytes of *in vivo* growth were collected from every mouse of 12, 14, 16, 18, 20, 22 days old (D12－D22; the *in vivo* group). The growth stage of oocytes collected in mice of 12, 14, 16, 18, 20, 22 days old corresponded to the oocytes collected from *in vitro* cultured follicles at day 2, 4, 6, 8, 10, 12, respectively.

Each oocyte was put into a drop of 0.5% pronase for 15 s to digest the zonal pellucida and was then rinsed three times in PBS. One single oocyte was put into 10 μL of lysis buffer, and was stored at －80 ℃ until assayed. RT-PCR reactions were carried out following the manufacturer's instructions using a mixture of poly (T) primers and Superscript Ⅲ reverse transcriptase enzyme in a volume of 20 μL to produce cDNA. The mixture was incubated at 25 ℃ for 25 min, 50 ℃ for 60 min and 70 ℃ for 15 min, and the solution was stored at －20 ℃ before half of the volume was assayed for β-actin, and the other half was assayed for GDF-9. The oligonucleotide primers for β-actin were 5′-TCGTGGGCCGCTCTAGGCAC-3′ 5′-TGGCCTTAGGGTTCAGGGGG-3′ and for GDF-9 was 5′-AGCAGAAGTCACCTCTACAATAC-3′ 5′-GTGTCGTTGAGATACAAGATGA-3′. The amplification profile of β-actin included an initial step of 94 ℃ for 2 min, followed by 30 cycles of dissociation at 95 ℃ for 30 s, annealing at 60 ℃ for 40 s and then extension at 72 ℃ for 30 s. Similarly the amplification profile of GDF-9 included an initial step of 94 ℃ for 2 min, followed by 35 cycles of dissociation at 95 ℃ for 30 s, annealing at 56 ℃ for 40 s then extension at 72 ℃ for 30 s. The amplified products were stored at －20 ℃. The PCR products were electrophoresised on a 2% agarose gel and an 100 bp DNA marker was used to show approximate sizes of the amplified fragments. Moreover, sequence analysis was

performed to confirm the identity of PCR products. The bands were quantified by OPIDI (AREA × OPTDM), the AREA and OPTDM were measured automatically by densitometry. The mRNA level was normalized on the basis of β-actin mRNA content. Therefore the GDF-9 mRNA was expressed as the ratio of GDF-9 to β-actin.

1.4 Statistical Analysis

The data were analyzed using the paired Student's *t*-test.

2 Result

Morphological changes were observed everyday during *in vitro* culturing of preantral follicles (Fig. 1): at D2 – 3, the follicles stuck to the bottom of the culture dishes; at D4, granulosa cells obviously increased in quantity; at D5 – 6, granulosa cells duplicated quickly and crossed over the follicle basal membrane, oocytes could not be seen clearly since they were veiled by the granulosa cells; at D7 – 10, a translucent antrum-like cavity appeared in the centre of the follicles, and became bigger with time. Fourteen hours after the simulation of HCG, mucified cumulus oocyte complexes (COCs) were seen outsides the follicles. When stripped of the cumulus cells MⅡ, MⅠ and GV oocytes could be seen. Three hundred and six preantral follicles were cultured for 12 days; 274 survived until D12, antral-like cavities were seen in 143 follicles and 155 MⅡ oocytes were obtained in total. The follicle survival, antrum formation and maturation rate was 89.5%, 51.8% and 56.6%, respectively. Follicles degenerated mostly at D4 when the follicle basal membrane ruptured and oocytes were split out of the follicles.

Oocytes were collected at different stages and a totae of 120 oocytes were assayed. After semi-quantitative RT-PCR, two kinds of products were obtained: 416 bp fragments of GDF-9 and 227 bp fragments of β-actin (Fig. 2). The OPIDI bands of PCR products were observed and the ratio of GDF-9 to β-actin was calculated (Tab. 1). At D2, the relative mRNA abundance of GDF-9 in mice oocytes collected *in vitro* was a little higher than that collected *in vivo*, but the difference was not significant ($P > 0.05$). At D4 and D6, the GDF-9 mRNA of *in vitro* oocytes decreased dramat-

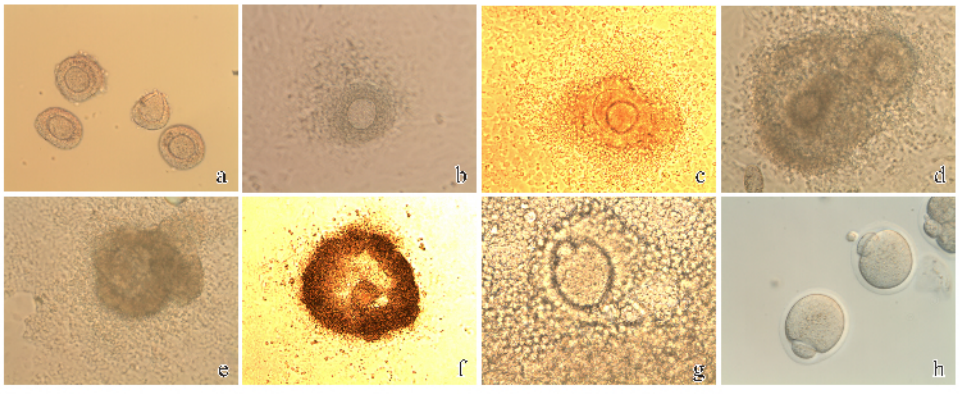

Fig. 1 Follicles cultrued *in vitro* for 2(a), 4(b), 6(c), 8(d), 10(e), 12(f) and 13(g) days, a – d ×200; e,f ×100; g ×400; h:MⅡ oocytes obtained from *in vitro* culture ×400.

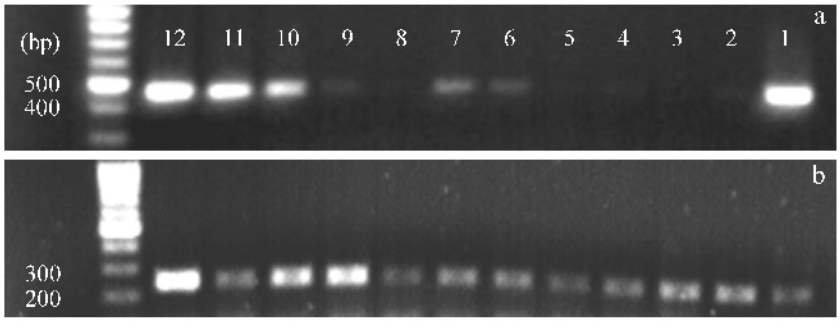

Fig. 2 RT-PCR analysis of GDF-9 (a) and β-actin (b) expression of oocytes grown *in vitro* (lanes 1 – 6, D2 – 12) and *in vivo* (lanes 7 – 12, D12 – 22)

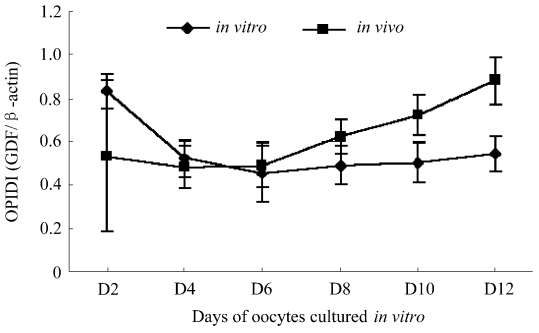

Fig. 3　OPIDI (GDF-9/β-actin) of oocytes grown *in vitro* and *in vivo* at different growing stages

ically, which was similar to the *in vivo* oocytes. At D8, D10 and D12, the relative mRNA abundance of GDF-9 of *in vitro* oocytes was significantly different from that of *in vivo* oocytes ($P < 0.05$) (Fig. 3).

3 Discussion

Obtaining mature oocytes after *in vitro* culturing of preantral follicles has been successful in many laboratories. We also obtained MⅡ oocytes after 12 days *in vitro* culturing of preantral follicles, with a maturation rate of 56.6%. The problem is that the developmental potential of the obtained oocytes is lower than the

Tab. 1　Relative mRNA abundance of GDF-9 in oocytes cultured *in vitro* and growing *in vivo*

	OPIDI	D2	D4	D6	D8	D10	D12
In vitro	GDF-9	28 650 ± 6 311	17 281 ± 1 236	15 370 ± 2 543	15 758 ± 3 371	15 843 ± 1 546	18 040 ± 2 001
	β-actin	34 601 ± 5 223	33 206 ± 2 700	34 331 ± 2 311	32 395 ± 4 849	32 012 ± 3 033	33 823 ± 2 997
	GDF-9/β-actin	0.83 ± 0.08	0.52 ± 0.09	0.45 ± 0.13	0.49 ± 0.09	0.50 ± 0.09	0.54 ± 0.08
In vivo	GDF-9	18 226 ± 2 207	15 861 ± 3 303	16 418 ± 4 120	21 688 ± 3 457	24 798 ± 3 652	29 858 ± 2 239
	β-actin	34 270 ± 4 897	32 900 ± 3 320	34 850 ± 1 982	35 309 ± 1 493	34 829 ± 2 009	34 033 ± 4 064
	GDF-9/β-actin	0.53 ± 0.35	0.48 ± 0.10	0.49 ± 0.10	0.62 ± 0.08*	0.72 ± 0.09*	0.88 ± 0.11*

* $P < 0.05$.

oocytes growing *in vivo*, which is demonstrated by a lower blastocyst rate (Eppig et al, 1989; Liu et al, 2002; Adam et al, 2004). Although there are still many ambiguous points on oocyte maturation, some experiments provide clues to clarify the points. In mice, a follicle diameter of 300 μm is the threshold for forming sinus cavities which is essential for oocyte maturation (Bishonga et al, 2001; Boland et al, 1993). Therefore the proliferation and differentiation of follicle cells is believed pivotal to the development of oocytes. However, in recent years, oocytes have evoked great interest for research. A series of experiments on vacuolated follicles and reorganized follicles infer that oocytes themselves control the growth and development of follicles (Eppig et al, 2002). The paracrine factor GDF-9 secreted by oocytes plays important roles in the intercommunication between oocytes and follicle cells. Although Kim et al (2004) had worked on oocytes matured *in vitro* and *in vivo* and found no difference of the GDF-9 gene expression between them, the GDF-9 oscillation at different growth stages of oocytes was still unknown.

We cultured preantral follicles of 10-day old mice for 12 days and traced the GDF-9 gene expression of the oocytes growing *in vivo* and *in vitro*. The relative abundance of GDF-9 mRNA varied as the follicles grew. At D12 of *in vivo* growth oocytes, the relative abundance of GDF-9 mRNA was high. At D14, the curve descended dramatically and reached the lowest point. Later the curve increased slowly. As we know, during this period of time, *in vivo* follicles morphologically changed; granulosa cells differentiated and the sinus cavity formed. We conferred that GDF-9 is related to the follicle construction. GDF-9 is involved in stimulating proliferation of granulosa cells and inhibiting their differentiation. When GDF-9 expression is high in oocytes, follicles grow. On the other hand, when GDF-9 expression is low, granulosa cells surrounding the oocytes differentiate into cumulus cells and mural cells and sinus cavities appear. Different growth stages correspond to different GDF-9 gene expression levels.

The GDF-9 oscillation curve of *in vitro* oocytes was different from *in vivo* oocytes. The relative abundance of GDF-9 was at a high level at D2 when the follicles stuck to the walls of dishes and began to grow. GDF-9 descended sharply at D4. Two days later, antrum-like cavities formed in cultured follicles. From D8 to D12, we observed significantly different GDF-9 gene expression between *in vitro* oocytes and *in vivo* oocytes. The relative abundance of GDF-9 stayed at a very low level *in vitro* until it increased at D12 (not significant), while GDF-9 *in vivo* increased dramatically after the antrum formation. Since the antrum formation stage is very important for oocyte maturation (Fair, 2003), the low GDF-9 expression during the antrum-like cavity for-

mation stage in oocytes cultured *in vitro* might be the reason for their low development potential.

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